TRANSCRIPTIONAL AND TRANSLATIONAL EXPRESSION OF A WILD TYPE BACTERIAL TOXIN GENE SEQUENCE IN TRANSGENIC SUGARCANE

TN CASSIM[1], BB MONOSI[1], GM MEYER[1], FC BOTHA[1,2], RS RUTHERFORD[1] AND BI HUCKETT[1,2]

[1] *South African Sugar Association Experiment Station, Private Bag X02, Mount Edgecombe, 4300, South Africa*
[2] *Institute for Plant Biotechnology, University of Stellenbosch, Private Bag X1, Matieland, 7602, South Africa*

Abstract

Genes from bacterial sources often confer characteristics that are seen as highly desirable for introduction into crops by genetic engineering. However, bacterial gene sequences are not always compatible with the molecular mechanisms operating in plants. In this work, expression of a truncated but otherwise unmodified bacterial toxin gene was examined in transgenic sugarcane clones. Molecular analysis of specific RNA transcripts and protein products, by Northern and Western blotting respectively, showed that RNA transcripts were characterised by significant premature polyadenylation triggered at specific points in the gene sequence, while protein levels were undetectable.

Introduction

In the genetic engineering of higher plants, the chosen transgene of interest is often bacterial in origin. Bacteria have evolved a diverse array of metabolic pathways and products not found in eukaryotic organisms, and the genes encoding those characteristics have the potential to add novelty to plant phenotypes. In addition, bacterial characters of interest are often single gene traits encoded by simple genes, suitable for cloning into small DNA vectors for delivery to the plant and subsequent integration into the plant genome. Examples of bacterial genes used widely in plant transformation are those encoding enzymes that effect herbicide resistance and those producing insecticidal proteins such as the endotoxins from strains of *Bacillus thuringiensis* (*B.t.*). A truncated native *B.t.* gene from *Bacillus thuringiensis* strain 234, isolated at Mount Edgecombe (Herrera *et al.*, 1994), has been used to produce a number of sugarcane transformants of varieties NCo310 and NCo376. The *B.t.* 234 toxin, of the CryIA(c) type, is particularly effective against the sugarcane stalk borer, *Eldana saccharina* Walker (Lepidoptera: Pyralidae). A subset of NCo310 plants confirmed as transgenic for the *B.t.* 234 gene has been the subject of gene expression studies at transcriptional (mRNA) and translational (protein) levels. Results of these expression analyses are reported here.

Materials and Methods

Sugarcane material

Various individual *B.t.* transformant plants and non-transgenic NCo310 were micropropagated to produce a number of clones of each type. These were maintained in the containment glasshouse at the South African Sugar Association Experiment Station (SASEX) at Mount Edgecombe using conventional pot fertilisation and automated watering regimes. Experimental material consisted of plants ranging from 500 mm to 2.5 m in height.

RNA extraction, RT-PCR and Northern analysis

Leaf tissue was the source of all RNA extracts used in this study. Third youngest leaves were randomly sampled from three NCo310 control plants and from three individual plants within each transformant line at each daily time point. Sampling was done at the same time each day (between 11h00 and 12 noon) and the same portion of the leaf removed in each case. Samples were pooled for each line, immediately frozen in liquid nitrogen and stored at -80°C. For reverse transcription-polymerase chain reactions (RT-PCR), DNA-free RNA was extracted using the SV Total RNA Isolation System (Promega), while for Northern analysis the RNeasy Extraction kit (Qiagen) was used to prepare larger amounts of total RNA. In each RT-PCR reaction, 1 µg RNA was used in a final volume of 50 µl in a single step procedure (Titan System, Boehringer Mannheim). RT-PCR products were analysed by electrophoresis in agarose gels (1%, w/v) and visualised by conventional ethidium bromide staining. RNA for Northern analysis (15 µg per sample) was fractionated through agarose (1,2%, w/v) in the presence of formaldehyde, and the resultant profiles checked by ethidium bromide staining for equality of loading and presence of undegraded ribosomal RNA bands before transfer to positively charged nylon membrane (Amersham) using the downward capillary blotting method of Chomczynski and Mackey (1994). Probe DNA was generated by PCR amplification from plasmid vector of a 1 850 bp fragment of the *B.t.* 234 gene using specific primers. *B.t.* gene amplification products were purified by agarose gel electrophoresis followed by excision of bands and column extraction (QIAquick, Qiagen). Isotopic labelling was by random

primed deoxynucleoside triphosphate incorporation (Megaprime, Amersham) using ^{32}P-dATP (Amersham) as signal component. Hybridization of probe to membrane and subsequent washes were at 65°C. Visualization of signal was by autoradiography using x-ray film (Hyperfilm-MP, Amersham).

Protein extraction and Western analysis

Samples (2 g) of plant material (leaf roll) were frozen in liquid nitrogen and ground in 38 ml of cold extraction buffer (50 mM K_2CO_3, pH 9,5, 100 mM KCl, 0,05% (w/v) Triton X100, 0,05% (w/v) Tween 20, 1 mM phenyl methyl sulphonyl fluoride and 1 mM iodoacetamide). Following centrifugation, the soluble protein concentration in each sample was estimated using the method of Bradford (1976) (BioRad) with bovine serum albumin (BSA) as the protein standard. Fifty µg of soluble protein per sample was loaded on to each lane and subjected to electrophoresis at 200 V through a 15% (w/v) SDS-PAGE gel (Schagger and von Jagow, 1987). Electroblotting to Hybond™-P membrane (Amersham) was performed in transfer buffer consisting of 25 mM Tris base, 192 mM glycine, and 20% (v/v) methanol (Towbin *et al.*, 1979). Following transfer, the membranes were blocked with SuperBlock™ (Pierce) overnight and then probed for 2h with rabbit polyclonal CryIA(c) antiserum at a 1:4000 dilution in 20ml TBST (50mM Tris-HCl, pH 7,5, 200 mM NaCl, 0,05% (w/v) Tween-20) containing 0,6 g BSA. The filters were then washed for 5 minutes, 3 times, in TBST and then probed with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) at a 1:1000 dilution in TBST for 30 min. The membranes were developed following three 5 minute washes in TBST by addition of nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (Boehringer-Mannheim) in alkaline phosphatase substrate buffer (100 mM Tris-HCl, pH 9,5, 100 mM NaCl, 50 mM $MgCl_2$). The reaction was stopped by washing the membrane in 100 mM EDTA.

Results and Discussion

Preliminary evidence for the production in the leaves, via transcription, of *B.t.* transgene mRNA was provided by RT-PCR analyses. Results (not shown) demonstrated that all transformants tested in this study were *B.t.* RNA positive, as

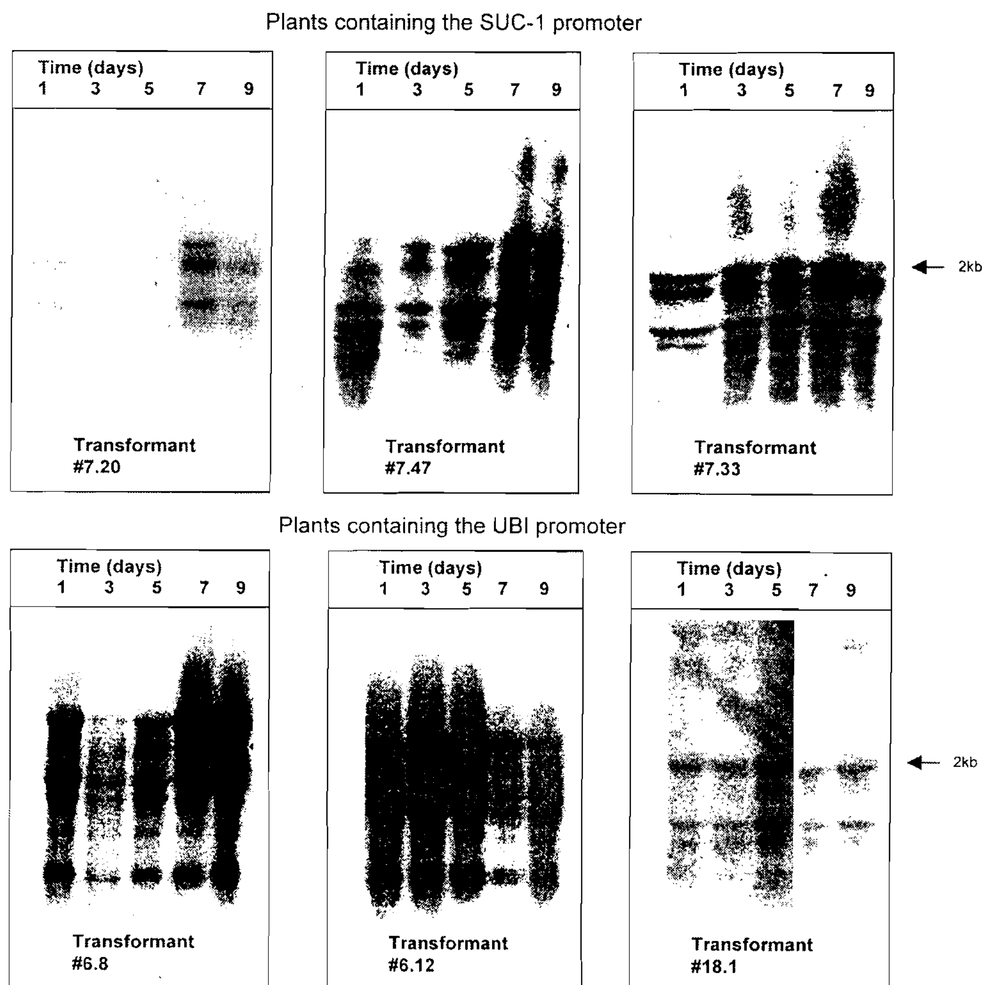

Figure 1. *B.t.* 234 gene transcripts in leaves of six different sugarcane transformants. Plant material was sampled for RNA extraction over a period of 9 days of normal glasshouse growth. Transcripts were characterised by Northern analysis using 15µg total RNA per sample (lane). The specific activity of the *B.t.* probe used was 1.9 x 10^9 cpm µg DNA^{-1}. Autoradiographic film was exposed to blots for 4 days at -80°C.

shown by unambiguous gel bands of the expected size in those samples and complete absence of bands in non-transgenic NCo310 (control) samples. One of the limitations of RT-PCR, however, is that detection of RNA is not quantitative, nor does it indicate the original *in vivo* size of the RNA species being amplified. For that reason, Northern analysis was undertaken to provide further information about the *B.t.* transcripts. Results of Northern analysis for the six transformants are shown in Figure 1. Parallel processing of the non-transformed (control) NCo310 resulted in a blank autoradiograph (not shown). Two characteristics of the *B.t.* transcripts are strikingly apparent. Firstly, there is a wide variation in overall band intensity (mRNA population density) between transformants. This most probably reflects the effect of transgene position in the genome, which is known to vary as a result of random integration following delivery. Positional effects on expression are well documented (Weising *et al.*, 1988). Results such as these for sugarcane serve to emphasise the importance of selecting suitable genotypes and phenotypes from among large populations of transformants during the genetic engineering procedure. Secondly, and more importantly, the *B.t.* mRNA shows an unusual multiple banding pattern in all cases, with consistent size species evident at approximately 2,0, 1,6, 1,2, 1,0 and 0,35 kb. The expected full length transcript (2,0 kb) does occur, but it is clear that a large proportion of mRNA molecules do not reach full length during transcription, rather being terminated at particular positions corresponding to the lengths of the four smaller species. Recently this phenomenon has been reported for the *B.t.* gene by researchers elsewhere (Diehn *et al.*, 1998). The practical implication is that transcription to full length mRNA is inefficient, leading to a likely reduction in translation and, ultimately, low levels of toxic protein.

Western (protein) blotting analysis of *B.t.* 234 transformed plants has shown that the CryIA(c) endotoxic protein is not detectable in even 50 µg of total soluble protein (Figure 2). Protein was extracted at pH 9,5, conducive to *B.t.* endotoxin solubility (Höfte and Whiteley, 1989). The lack of signal illustrated in Figure 2 is typical of results obtained for many *B.t.* 234 transformed lines, and for a range of different tissues, including leaf roll, leaf, tissues sampled after a wounding stimulus and callus. Although the particular plant lines tested for protein (Figure 2) differ from those analysed for mRNA (Figure 1), the results support the general conclusion that there is a likely causal link between the low levels of full length transcript and undetectable levels of translation product demonstrated. Premature termination of wild type *B.t.* gene transcripts have been shown by Green and coworkers (Diehn *et al.*, 1998; De Rocher *et al.*, 1998) to be associated with mRNA instability and to be a definitive cause of poor expression in higher plant systems such as *Nicotiana tabacum and Arabidopsis.*

Dietary bioassays quantifying the effect of purified *B.t.* protein on *E. saccharina* are being conducted at present, one aim being to determine whether toxicity is evident at concentrations of CryIA(c) that are undetectable by Western

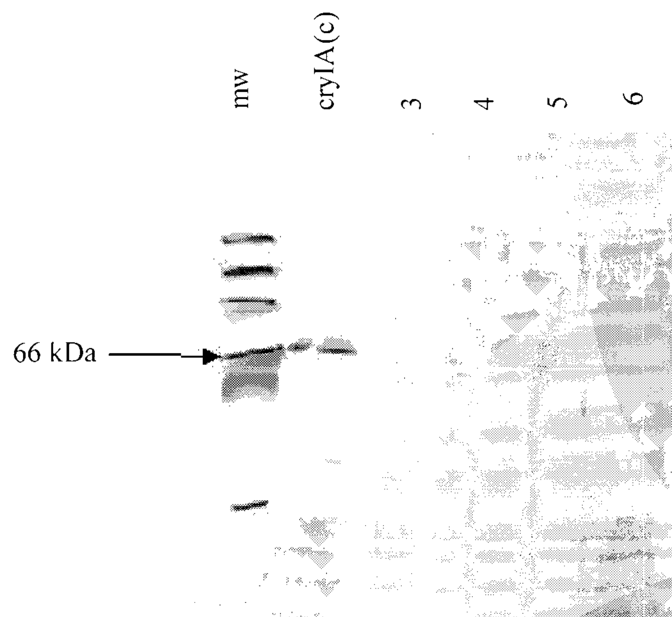

Figure 2. Typical protein blot showing position of purified truncated *B.t.* Cry IA (c) protein with respect to molecular weight markers (mw) and transformed plant protein extracts. Lanes 3-5: 50 µg of total soluble protein of *B.t.* 234 positive lines #7.01, #7.05 and #7.44 respectively. Lane 6: 50 µg of total soluble protein from an untransformed control plant.

blotting (less than 1 ng per 50 µg lane load, i.e. 0,002%). In addition, a well replicated pot trial bioassay has been established under shade house conditions for all 59 *B.t.* 234 transformed lines obtained at SASEX (Malcolm Keeping, Entomology department). This will involve egg deposition on plants that have been pot grown for 17-18 weeks and then stressed. Conclusive resistance data should be provided by this trial in due course.

The results of this study illustrate an important constraint suffered by some foreign transgenes in plants. Although clear evidence for premature polyadenylation has not been documented until recently (Diehn *et al.*, 1998) it has long been recognised that potential polyadenylation signals may be found in bacterial genes, together with other AT-rich motifs and rare codons which are inhibitory to expression in the plant environment. Such impediments have been the rationale behind gene resynthesis projects, in which gene sequences have been altered to suit plant usage while conserving the precise structure of the encoded protein. One example is the resynthesis of the *B.t.* gene itself (Perlak *et al.*, 1990, 1991), which, when applied in the genetic engineering of cotton, was shown to result in a 100-fold increase in expression compared with wild type sequences. Resynthesised *B.t.* genes are now the mainstay of commercial genetic engineering strategies for insect resistance, and form the basis of the current *B.t.* biotechnology programme at SASEX.

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ARE SELF-TRASHING VARIETIES OF SUGARCANE RESISTANT TO THE STALK BORER *ELDANA SACCHARINA* (LEPIDOPTERA: PYRALIDAE)?

MG KEEPING AND GW LESLIE

South African Sugar Association Experiment Station, P/ Bag X02, Mount Edgecombe, 4300, South Africa

There are indications that pre-trashing of sugarcane reduces infestation by *Eldana saccharina* Walker, by removing egg-laying sites for female moths or exposing eggs and neonate larvae to predators and desiccation (Carnegie and Smaill, 1982; Leslie, 1989). The self-trashing habit of certain varieties, in which dead leaf material falls naturally from the plant (leaving the stalk largely exposed), may achieve the same effect. Consequently, self-trashing varieties could be less severely attacked by *E. saccharina*. Cochereau (1981; cited in Leslie, 1990) reported that this was so for the self-trashing variety Ragnar, when compared with other varieties grown in the Ivory Coast. Self-trashing may also have other advantages, e.g. reduced lodging where green cane harvesting is practised (Cock *et al.*, 1997). On the other hand, for the sugarcane borer *Chilo sacchariphagus indicus* (Kapur) (Lepidoptera: Pyralidae), tight leaf sheaths present a significant barrier for access to the stalk by early instar larvae (David and Joseph, 1982).

Two trials were established with the following objectives: (1) to test the hypothesis that self-trashing varieties of sugarcane enjoy significantly less damage by *E. saccharina* than non-self-trashing commercial varieties; (2) to investigate the effect of artificially loosened or tightened trash on borer survival and crop damage in resistant and susceptible commercial varieties. Trial 1, containing seven self-trashing clones and five commercial varieties, was planted at the Experiment Station's La Mercy research farm in October 1992. Trial 2, containing five self-trashing clones and five commercial varieties, was planted at the Mount Edgecombe research farm in June 1994.

In both trials, trash of the commercial varieties N11 (susceptible) and N21 (resistant) was manipulated in treatment plots to produce tight trash (T/T) and loose trash (L/T) characteristics. In Trial 2, trash manipulation also included the commercial varieties N17 (intermediate) and N22 (susceptible). Both trials contained untreated control plots of the above varieties. Cane stools in all plots were then exposed to *E. saccharina* through release of 10 pairs of females and males into cages placed over the stools for two days. Trials were harvested after 500-day degrees had accumulated from the date of moth release. At this stage larvae were fifth to sixth instar (Way, 1995). Data collected included length of stalk bored and internodes bored, and numbers and mass of *E. saccharina* larvae and pupae. The data were used in a formula producing a single value for each treatment, expressed as the per cent of the overall trial mean for this same variable (with the latter equal to 100%).

Results indicate that the self-trashing habit provided no clear advantage in terms of resistance to *E. saccharina*. There were significant differences in susceptibility to the borer among the self-trashing selections (Table 1), but the source/s of these differences could not be identified or ascribed to the self-trashing habit. In Trial 1, four selections (89E0033, 89E0042, 89E0509, 89E0033) showed susceptibility levels comparable with the susceptible controls N11 and N22 (Table 1), and in Trial 2 the same was observed for two selections (90W0074, 90W1147). Other than 90E1297 (in Trial 2), none of the self-trashing clones approached the resistance level of N21 (Table 1), the expected trend if self-trashing confers some protection against *E. saccharina*. For all crops in both trials, the mean level of susceptibility for the self-trashing selections was higher than the trial mean (i.e. >100%).

Manipulation of trash in the commercial varieties produced contradictory results, depending on variety, trial and crop. N11-loose trash displayed greater damage than N11-tight trash and untreated N11 in the plant crop of Trial 1, but this was reversed in both crops of Trial 2 (Table 1). N21-tight trash, on the other hand, displayed greater levels of damage than N21-loose trash in both crops of both trials and untreated N21 in both crops of Trial 1 and the ratoon crop of Trial 2 (Table 1). In Trial 2, N17-loose trash exhibited less damage than untreated N17 in both crops, while N22-tight trash exhibited less damage than untreated N22 in the plant crop but greater damage in the ratoon crop. Although this suggests that varieties show a differential response to trash manipulation, the interaction between variety and trash manipulation was non-significant (Table 1).

If the self-trashing trait conferred resistance, self-trashing clones and loose trash treatments should consistently have exhibited lower levels of damage than untreated controls and tight-trash treatments. The average effects of loose and tight trash treatments were significant only in Trial 2, where they reduced and increased borer damage/abundance, respectively (Table 1). However, only one effect was significant in each crop of this trial (Table 1).

In general, spatial variation in the trial was high, especially

Table 1. Summarised results of plant and ratoon crops of self-trashing trials 1 (La Mercy) and 2 (Mount Edgecombe). Mean values are overall percentages of the trial mean (=100%) calculated from four variables measuring stalk damage and borer performance.

Treatment means & statistics	Trial 1 - Plant	Trial 1 - Ratoon	Trial 2 - Plant	Trial 2 - Ratoon
Means:				
89E0033	193	125	-	-
89E0042	287	152	-	-
89E0233	89	88	-	-
89E0259	71	74	-	-
89E0509	99	141	-	-
89E0795	83	67	-	-
89E1253	92	126	-	-
90W0074	-	-	57	106
90W1053	-	-	102	80
90W1207	-	-	40	40
90W1147	-	-	141	296
90E1297	-	-	7	20
Selections mean	131	116	110	108
N17	33	69	104	82
N17 Loose trash	-	-	46	56
N22	83	133	210	77
N22 Tight trash	-	-	165	183
NCo376	73	81	153	85
N11	131	129	187	115
N11 Tight trash	114	154	194	182
N11 Loose trash	199	155	114	150
N21	22	33	30	37
N21 Tight trash	30	39	30	56
N21 Loose trash	2	34	20	36
Tight trash effect	72	96	130	140
Loose trash effect	101	94	60	81
LSD (p=0,05)	78,6	44,4	69,9	69,8
CV	68,0	38,4	60,6	60,5
F values (* significant at p=0,05):				
Selections	8,32*	4,65*	4,50*	19,87*
Control varieties	2,44	7,30*	8,44*	1,26
Tight trash effect	0,03	1,36	0,38	10,06*
Loose trash effect	0,77	1,02	5,44*	0,02
Variety x T/T	0,20	0,36	0,64	1,56
Variety x L/T	2,53	0,65	0,88	0,76

for *E. saccharina* numbers and weight, where edge effects were pronounced in spite of the presence of guard rows on all sides. Normally, the lattice square trial design controls for variation in both rows and columns (Murdoch[1], personal communication).

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1 M. Murdoch, Head Biometry Dept, SASEX, Mt Edgecombe

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FIELD SCREENING OF SUGARCANE VARIETIES FOR RESISTANCE TO THE STALK BORER, *ELDANA SACCHARINA* (LEPIDOPTERA: PYRALIDAE)

MG KEEPING

South African Sugar Association Experiment Station, P/Bag X02, Mt Edgecombe, 4300, South Africa

To date, routine screening of unreleased varieties (from stages 4 and 5 of the sugarcane selection programme) for resistance to *Eldana saccharina* Walker has been conducted in shade house trials where cane is artificially inoculated with *E. saccharina* eggs (Nuss, 1991; Nuss and Atkinson, 1983). Although this screening method ensures equal exposure of all test varieties to larvae of the borer, and excludes many uncontrolled environmental variables inherent in field trials, it also precludes moth choice of varieties for oviposition, as a potential resistance mechanism (e.g. Sosa 1990). Thus the total field resistance of test varieties should include information on their performance against *E. saccharina* in the field as well as in shade house trials. Field performance data are currently obtained from surveys for damaged stalks in observation, primary and secondary plant breeding trials at the Experiment Station's Mtunzini, Shakaskraal and La Mercy research farms. However, low to mild *E. saccharina* infestations, such as those experienced over the past three years, do not provide an adequate test of resistance, throwing doubt on the value of the data obtained.

In light of the above, two field trials, each containing 36 varieties, were established to test: (a) discrimination of varietal differences in resistance to *E. saccharina* under field conditions, (b) repeatability of resistance ratings between successive crops, and (c) whether field and shade house screening of varieties produced comparable resistance ratings. Trial 1, containing 24 unreleased clones and 12 commercial controls, was planted at La Mercy research farm in August 1993 and harvested annually over four crops. Trial 2, containing 28 unreleased clones and eight commercial controls, was planted at the Mount Edgecombe research farm in December 1995 and consisted of one 18 month old crop. This trial and a shade house trial performed during the same year contained the same varieties.

For Trial 1, *E. saccharina* infestation levels were enhanced through inoculating sorghum and/or maize, intercropped with the cane, with eggs when the cane was seven months old. Moths emerging from the intercrop subsequently oviposited in the adjacent rows of cane when the latter was approximately nine months old. White and Legendre (1991) used similar methods to intensify the level of borer (*Diatraea saccharalis*; Lepidoptera: Pyralidae) pressure in sugarcane in Louisiana. Trial 2 was infested through direct inoculation with 500 eggs per stool when the crop was 15 months old. Higher damage levels were achieved using the inoculated intercrop method than through direct inoculation (10,8% vs. 2,8% internodes damaged, respectively). Each crop in Trial 1 was harvested at 12 months and Trial 2 was harvested at 19 months. Data collected included length of stalk bored and internodes bored, and numbers and mass of *E. saccharina* larvae and pupae. Length of stalk bored and borer mass were not recorded for the third ratoon of Trail 1.

Discrimination of varietal differences (i.e. Degree of Genetic Determination or DGD) in resistance was high in both trials (Table 1), except for the third ratoon of Trial 1, where damage and borer numbers were reduced, probably due to the poor nutritional status of the crop. Borer numbers and damage increased in Trial 1 from the plant crop to the second ratoon (Table 1). Ratings were the same between at least two crops for 86% of varieties in Trial 1. Broad resistance categories (i.e. susceptible, intermediate, resistant) were matched between Trial 1 and Plant Breeding (PB) records for 76% of 34 varieties for which PB records were available. Exact resistance ratings on a 1-9 scale differed between the Trial 1 mean (n=4 crops) and PB records by two units for only eight of the 34 varieties; ratings for the remaining 26 varieties were either the same or differed by one unit. Broad resistance categories were matched between 69% of the 26 test clones included in Trial 2 and the 1995 shade house trial; exact ratings differed by two or more units for eight of the latter.

The results indicate that field trials, using either direct inoculation with *E. saccharina* eggs or infestation from an inoculated intercrop, can incur adequate borer infestation and crop damage to allow for reliable estimation of resistance levels in test clones. However, it is clear that ratings derived from at least three successive crops were required to produce satisfactory agreement with resistance ratings established over several years of shade house testing and/or PB field surveys for *E. saccharina*. Observed differences in the resistance rating for the same variety from field and shade house trials may be due to the effect of antixenosis acting on moth varietal choice in field trials, a mechanism for which there is no allowance in shade house trials.

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Table 1. Trial means and degree of genetic determination for variables measured in *Eldana saccharina* field Trial 1 (La Mercy) and Trial 2 (Mount Edgecombe).

Trial & crop	Borer numbers per plot (CV%)	Per cent stalks damaged (CV%)	Per cent internodes damaged (CV%)	Degree of Genetic Determination (DGD)*			
				Length damaged	Internodes damaged	Borer numbers	Borer mass
Trial 1:							
Plant	6,5 (64,3)	53,6 (21,9)	10,8 (30,2)	0,62	0,64	0,51	0,52
R1	12,2 (51,1)	78,0 (12,7)	17,8 (30,5)	0,89	0,86	0,64	0,56
R2	78,8 (45,5)	85,0 (10,4)	22,0 (28,6)	0,82	0,82	0,75	0,71
R3	6,6 (91,3)	45,1 (25,7)	5,8 (39,5)	-	0,71	0,56	-
Trial 2:							
Plant	7,4 (79,9)	26,0 (42,6)	2,8 (60,9)	0,83	0,88	0,85	0,82

*DGD (selections only) = (F-1)/F. The closer the DGD value is to 1,0, the higher the discrimination of heritable varietal differences.

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GLASSHOUSE TESTS FOR OVIPOSITIONAL ANTIXENOSIS OF SOUTH AFRICAN SUGARCANE VARIETIES TO *ELDANA SACCHARINA* (LEPIDOPTERA: PYRALIDAE)

LY MABULU[1,2] AND MG KEEPING[2]

[1]*Department of Zoology, University of Fort Hare, P/Bag X1314, Alice, 5700, South Africa*
[2]*South African Sugar Association Experiment Station, P/Bag X02, Edgecombe, 4300, South Africa*

Plant resistance is an important management strategy for stalk borers in several sugarcane growing areas of the world (Meagher *et al.*, 1996). Antixenosis is one of three resistance mechanisms employed by plants, which prevents or reduces colonisation by adult and immature insects. In the case of antixenosis to oviposition, resistance may derive from plant characteristics that either fail to stimulate or inhibit oviposition (Panda and Khush, 1995).

Glasshouse experiments were conducted to test for ovipositional antixenotic resistance of six commercial sugarcane varieties to the stalk borer *Eldana saccharina* Walker. Three trial designs (multiple-variety, paired-variety, and no-choice) using plants and moths placed in mesh cages measuring 2 m x 2 m x 2 m, provided data on number of eggs laid by female moths on each variety. In the multiple-variety choice tests, four plants of each of the sugarcane varieties N11, N14, N20 and N21 (two susceptible and two resistant, respectively) were placed in a cage in randomised positions. Fifteen newly emerged female and five male moths were released from the centre of the cage and egg laying allowed to proceed for 72 hours (three days).

In the paired-variety choice experiments, four plants of each of two varieties in the combinations N21+N14, N12+N14, N21+N16 and N12+N16 were placed in randomised positions in a cage. In each combination, a resistant variety was paired with a susceptible variety, giving the moths a simpler (binary) choice than that presented to them in multiple-variety trials. Numbers of moths released and period for oviposition were as for multiple-choice trials. In the no-choice tests, eight plants of the same variety (each of N11, N12, N14, N16, N20, N21) were presented to the moths for 72 hours; five newly emerged females and five males were released in the cage.

All experiments were replicated four times. For each trial, plants with a similar quantity of trash were used and their green leaves trimmed to produce similar plant height. At harvest the following measurements were taken: number of egg batches, positions of egg batches, number of eggs per batch, stalk lengths, internode diameters (10 cm above the stalk base) and trash mass (after oven drying at 60°C for 48 hours). Stalk length and internode diameters were measured as estimates of plant size which, together with quantity of trash (Leslie, 1990), may influence the numbers of eggs laid.

Other than on a single plant, where eggs were laid on green leaf material, all eggs in all experiments were laid on dead leaves and especially on curled dead leaf blades (Table 1).

In all three experiments, numbers of eggs and egg batches did not differ significantly between varieties at the 5% level. However, in no-choice experiments the mean number of eggs per batch differed significantly between varieties ($H=12,4$; $p<0,03$; Kruskal-Wallis ANOVA). In multiple-variety choice experiments, a significant correlation was obtained, using pooled data across varieties, for number of eggs versus internode diameter ($r=0,993$; $p<0,01$). This suggests that number of eggs laid may be related to plant size, although egg number and stalk length were not significantly correlated ($r=0,803$; $p>0,05$). Number of eggs and trash mass were not significantly correlated ($r=0,538$; $p>0,05$), suggesting that additional trash did not stimulate oviposition in the young plants used in this study.

Of the sugarcane varieties offered to the moths for oviposition, N21 received the highest number of eggs in all three experiments (Table 2), contrary to expectation as this is a resistant variety. In multiple-variety choice tests, the resistant variety N20 received the second highest number of eggs (25% of the total; Table 2), while in no-choice tests it received the least. Varieties N11, N12, N14 and N16 had variable numbers of eggs in the experiments where they were used. In general, the results show that for the varieties tested there was no consistent trend in egg-laying preference of *E. saccharina,* whether the moth had a choice or not. Moreover, coefficients of variation (CV) were high for egg numbers and egg batches in all three experiments (Table 2). The number of eggs within replicates was also inconsistent for all varieties, although N21 had the highest number of eggs when replicates were combined in all three experiments. In the paired-variety and no-choice experiments there was probably heavy predation by ants and spiders, as these were frequently seen on the plants and some plants carried no eggs at all. Predator exclusion is an important consideration for future experiments.

Ovipositional (antixenotic) resistance was not correlated with smaller stalk borer (*Diatraea saccharalis*; Lepidoptera: Pyralidae) populations in sugarcane (Kyle and Hensley, 1970); and work by Tucker (1933) and Fuchs and Harding

Table 1. Number of *Eldana saccharina* eggs found on different parts of the sugarcane plant and percentage (in parentheses) of total number of eggs laid on each plant part, in each experiment.

Experiment	Egg positions							Total
	Curled leaf blade	Midrib	Between stalk and sheath	Crevices on leaves	Curled leaf sheath	Rotten leaf sheath	Leaf ligule	
Multiple choice	1 883 (34)	313 (77)	319 (14)	0	268 (19)	75 (22)	31 (72)	2 889
Paired choice	1 219 (22)	43 (11)	637 (28)	23 (24)	73 (5)	121 (36)	0	2 116
No choice	2 443 (44)	51 (13)	1 337 (58)	72 (76)	1 097 (76)	142 (42)	12 (28)	5 154
Total	5 545	407	2 293	95	1438	338	43	10 159

Table 2. Mean number of *Eldana saccharina* eggs and egg batches found on each variety, coefficients of variation (CV), and percentage of total number of eggs and egg batches laid in each experiment.

Experiment	Variable	N11			N12			N14			N16			N20			N21			Total eggs
		Mean	CV	% of total eggs	Mean	CV	% of total eggs	Mean	CV	% of total eggs	Mean	CV	% of total eggs	Mean	CV	% of total eggs	Mean	CV	% of total eggs	
Multiple-choice	No. of eggs	31,9	186	19	-	-	-	40,7	161	23	-	-	-	45,4	123	25	62,6	110	35	2 889
	No. of egg batches	1,0	150	-	-	-	-	1,7	124	-	-	-	-	1,1	136	-	2,9	80	-	
Paired-choice	No. of eggs	-	-	-	31,2	195	21	34,2	147	27	20,1	192	18	-	-	-	14,8	223	34	2 116
	No. of egg batches	-	-	-	1,2	158	-	1,0	130	-	0,5	160	-	-	-	-	0,7	200	-	
No-choice	No. of eggs	30,8	169	24	21,5	220	13	25,8	160	16	17,8	256	11	14,4	190	5	40,3	162	31	5 154
	No. of egg batches	1,2	142	-	0,8	213	-	1,0	160	-	0,7	257	-	1,0	150	-	1,6	138	-	

Dashes indicate variety was not used in that experiment and/or value not applicable (numberof batches).

(1978) indicated no ovipositional preference of this species among sugarcane varieties in the USA. More recently, however, leaf pubescence was found to be associated with both ovipositional and larval antixenotic resistance against *D. saccharalis* (Sosa, 1988; 1990; Sosa *et al.*, 1997). Meagher *et al.* (1996) performed choice and no-choice tests on different cultivars (NCo310, CP 70-321 and CP 70-324) of Texas sugarcane against *D. saccharalis* and *Eoreuma loftini* (Lepidoptera: Pyralidae). In their no-choice tests, individual comparisons showed no significant differences in number of eggs laid between cultivars. However, there were trends for NCo310 to carry more eggs per plant than CP 70-321 and CP 70-324, and CP 70-324 to carry more eggs than CP 70-321.

Results of the present study using South African varieties indicate that ovipositional antixenosis to *E. saccharina*, at least in young sugarcane (5 months old), is probably not an important resistance mechanism.

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